Inhibition of the CyclinD1 promoter in response to sonic hedgehog signaling pathway transduction is mediated by Gli1

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Abstract. Medulloblastoma (MB) is the most common malignant tumor of the central nervous system in children. Accumulating evidence suggests a major role for the activation of the sonic hedgehog (SHH) signaling pathway in the development of MB cells; however, the mechanisms underlying the effect of this pathway on tumor survival and growth remain poorly understood. The Gli family zinc finger 1 (Gli1) transcription factor is considered as a mediator of the SHH signaling pathway in MB cells. Therefore, the present study investigated whether the SHH signaling pathway promotes the apoptosis of MB cells via downregulation of Gli1. GANT61, a novel Gli1 inhibitor, is known to have an *in vitro* activity against tumors. In the current study, Daoy cells were treated with different concentrations of GANT61 for 24 h, and the effect on cell proliferation was assayed by cell counting kit-8 assay. In addition, the cell cycle progression and apoptosis were assayed by flow cytometry analysis and hematoxylin-eosin (HE) staining. The effects of GANT61 treatment on SHH signaling pathway at the mRNA level were assayed by polymerase chain reaction (PCR). To further elucidate the inhibitory effects of GANT61 on the expression of Gli1 and CyclinD1, their protein levels were examined by western blot and immunofluorescence. The results indicated that GANT61 significantly inhibited the proliferation of Daoy cells in a dose-dependent manner, compared with the control group (P<0.05). HE staining revealed that cells had increasingly abnormal protuberance with increasing GANT61 concentration. Flow cytometry analysis also demonstrated that GANT61 induced G1/S arrest and apoptosis of Daoy cells in a dose-dependent manner (P<0.05). Gli1 and CyclinD1 mRNA expression levels were downregulated by GANT61 treatment (P<0.05); similarly, their protein levels were downregulated by GANT61 treatment in a dose-dependent manner (P<0.05). In conclusion, Gli1 expression was significantly associated with CyclinD1 expression in MB. These data demonstrated that Gli1 is an important mediator of the SHH pathway activity in MB, and may be a novel agent for use in combined chemotherapeutic regimens.

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Introduction

Medulloblastoma (MB) is the most common pediatric malignant brain tumor, and has a poor clinical outcome (1,2). With currently available multimodality therapies, including surgery, radiotherapy and chemotherapy, numerous children have a favorable prognosis; however, the majority of patients suffer from considerable long-term disabilities and morbidity following aggressive multimodal therapy (3-5). Attempts to further improve the outcomes have been restricted by the cytotoxicity of conventional medication and the nature of the disease. Therefore, an increased understanding of the mechanisms underlying MB is crucial in the development of novel therapeutic approaches.

Aberrant activation of the sonic hedgehog (SHH) signaling pathway has been implicated in the development of MB (6-8). The Gli family zinc finger 1 (Gli1) transcription factor is considered to be a mediator of the SHH signaling pathway in MB, although its tumorigenic nature and its relative contribution to tumorigenesis remain poorly understood (9).

CyclinD1 is a key protein in the cyclin family that regulates the G1/S transition and is highly expressed in multiple types of tumors (10,11). This protein is regulated by a complex system of signal transduction pathways (12,13). CyclinD1 expression is known to be regulated by Gli1 in MB. Furthermore, GANT61 is a specific Gli1 inhibitor, which has been shown to inhibit the DNA binding activity of Gli1 by binding to the zinc-finger domain (14-16).

In order to examine the role of Gli1 in MB, our previous studies screened for genes preferentially regulated by Gli1 in MB cells (17,18). CyclinD1 plays important role in tumor

proliferation, and thus the expression of CyclinD1 was investigated in MB cells.

Materials and methods

Reagents and antibodies. GANT61 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (DMSO) and stored at -20˚C until required for use. The final DMSO concentration in all cultures, including the vehicle control groups, was 0.1% in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA). Fetal bovine serum (FBS) and 0.25% trypsin/EDTA were purchased from Gibco (Thermo Fisher Scientific, Inc.). The hematoxylin and eosin (HE) staining kit (G1060) was purchased from SuoLaibao Technology Co., Ltd. (Beijing, China), and the FITC-Annexin V kit from Abcam (ab14150; Cambridge, MA, USA). The cell counting kit-8 (CCK-8) assay for cell proliferation analysis was purchased from Dojindo Chemical Research Institute (Tokyo, Japan), while the PrimeScript RT Master Mix and reverse transcription (RT) kit (RR014A) was obtained from Takara Bio, Inc. (Shiga, Japan; PrimeScript RT Master Mix). In addition, SYBR Green I was purchased from Beijing Noble Ryder Technology Co., Ltd. (Beijing, China). Antibodies against Gli1 (ab49314) and CyclinD1 (ab187364) were acquired from Abcam, while β-actin antibody (AP0060) was purchased from Bioworld Technology, Inc. (Louis Park, MN, USA). The secondary antibody of Gli1 (BL003A) and CyclinD1 (BL001A) were acquired from Biosharp (Wuhan, China) (19).

Cell culture. Daoy, an MB cell line, was purchased from ATCC (Manassas, VA, USA). The Daoy cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (500 ml; Gibco), 100 μg/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) at 37˚C with 5% CO_2. Prior to each experiment, trypan blue staining (Sigma-Aldrich) was used to define the cell vitality. The cell activity was determined to be >98%.

Cell proliferation analysis. CCK-8 assay was performed to investigate the cell proliferation, according to the manufacturer's instructions of the kit. Briefly, Daoy cells in exponential growth phase were pipetted into single cells following trypsin digestion. Cells were seeded in a 96-well plate at a density of 8×10^3 cells/well. RPMI 1640 medium containing 10% FBS was used to culture the cells for 24 h prior to replacing with serum-free medium. Next, the cells were starved for 6 h and then incubated in RPMI 1640 medium supplemented with 1% FBS. The cell culture groups included three groups treated with different concentrations of GANT61 (10, 20 and 40 μM) and a negative untreated control group with normal growing cells, while wells with no cells acted as the blank control. A total of six replicates per group were investigated. The cells were continually cultured in the incubator for a further 24 h before the culture medium was discarded. Subsequently, 100 μl fresh RPMI 1640 medium and 10 μl CCK-8 solution were added into each well. The cells were placed in the incubator to avoid light exposure, and the absorbance at 450 nm (A_{450}) was measured at 0.5, 1, 2 and 4 h, with a Bio-Rad 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).. The proliferation inhibition rate was calculated as follows: Proliferation inhibition (%) = [A_{450} (negative control group) - A_{450} (GANT61-treated group)] / A_{450} (negative control group) x100%.

HE staining. Daoy cells in the exponential growth phase were digested into a concentration of 1×10^6 cells/ml, added to glass coverslips and cultured for 24 h in an incubator. The medium was replaced, followed by addition of different concentrations of GANT61 (10, 20 and 40 μM), while the group without GANT61 treatment served as the control. Subsequently, the cells were extracted after culturing for 24 h, washed with phosphate-buffered saline (PBS) for three times and fixed in 4% paraformaldehyde for 60 min, followed by washing three times with PBS. The cells were then stained with hematoxylin for 5 min and washed by tap water. Following incubation in differentiation buffer for a few seconds and washing with water, eosin was added for 10 min. After washing with tap water, the stained sample was dehydrated, sealed and prepared for microscopic observation.

Flow cytometry. In order to investigate the cell cycle progression, flow cytometry analysis was performed using the FITC-Annexin V kit, according to the manufacturer's instructions. Briefly, 2×10^4 cells were transferred into 10-ml centrifuge tubes, and centrifuged for 5 min at 250-500 x *g* at 4˚C. After the culture medium was discarded, cells were washed once with the binding buffer and centrifuged for 5 min at at 250-500 x *g* at 4˚C. The final concentration of 1 μg/ml propidium iodide (PI) with FITC-Annexin V (included in the kit) was dissolved in incubation buffer. Resuspended cells were labeled in the dark for 10-15 min with 100 μl solution buffer at room temperature. Cells were then precipitated by centrifugation at at 250-500 x g at 4˚C for 5 min and washed with incubation buffer. The sample was then incubated at the 4˚C for 20 min in the dark without vibration. Detection and quantification of apoptotic cells was obtained by flow cytometry. This test was performed according to the manufacturer's instructions

RT-polymerase chain reaction (PCR) array analysis. Daoy cells were seeded in RPMI 1640 medium supplemented with 10% FBS, followed by exposure to different concentrations of GANT61 for 24 h, while the control was not treated with any GANT61. Total RNA was extracted from the cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) after 24 h, according to the manufacturer's instructions. The total RNA extracted was then treated with the PrimeScript RT Master Mix for removal of contaminating DNA and for reverse transcription into cDNA. Briefly, Primers specific for each of the signaling molecules were designed using NCBI/Primer-BLAST and used to generate the PCR products. The following primers were used: GLI1-Forward: 5'-GGG AGGAAAGCAGACTGACT-3'; GLI1-Reverse: 5'-TGGAGA GGTCTTCAGTGCTG-3'; CyclinD1-Forward: 5'-GCATGT TCGTGGCCTCTAAG-3'; CyclinD1-Reverse: 5'-CGTGTT TGCGGATGATCTGT-3'; GAPDH-Forward: 5'-CTCTCT GCTCCTCCCTGTTC-3'; GAPDH-Reverse: 5'-CAATCT CCACTTTGCCACTGC-3'. Target sequences were amplified

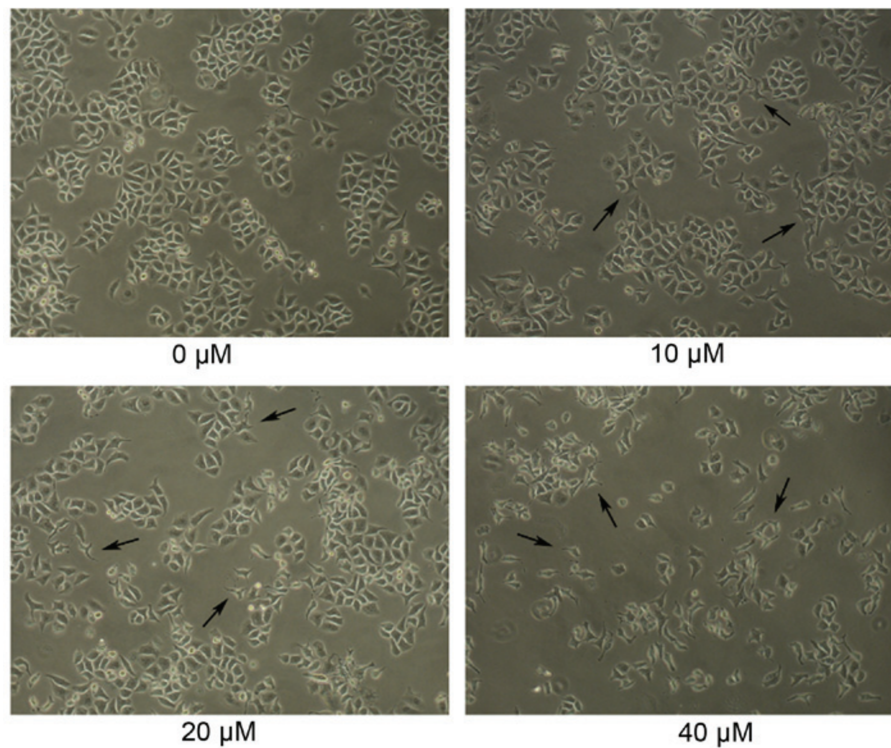

Figure 1. Morphological changes of GANT61-treated Daoy cells, as observed by inverted microscopy (magnification, x100). Normal adherent cells were intercellular tight, and their shapes were rectangular or triangular. However, Daoy cell groups treated with increasing concentrations of GANT61 demonstrated an evidently decreased number of cells, morphological changes and diversity.

at 95˚C for 1 min, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. GAPDH was used as endogenous normalization control. Subsequently, the samples were investigated by PCR array. Data were analyzed by the ΔΔCq method to determine the mRNA expression levels, as previously described (20,21). The experiment was performed in triplicate and repeated three times.

Western blot analysis. Daoy cells were synchronized in RPMI 1640 medium with 10% FBS, followed by exposure to different concentrations of GANT61 for 24 h, while the control was not treated with any GANT61. The protein profile in the samples was examined by western blot analysis. Briefly, cells were collected and washed three times with PBS. Next, the cells were lysed in fresh radioimmunoprecipitation assay protein lysis buffer containing phenylmethylsulfonyl fluoride (ratio, 100:1) on ice. The total protein concentration was determined by the BCA method (ab102536; Abcam). Following separation by 10% SDS-PAGE, the samples were transferred to polyvinylidene difluoride films. Protein blots were visualized by Ponceau S staining. The films were subsequently blocked with 5% non-fat milk for 2 h at room temperature. Anti-Gli1 (1:500) and anti-CyclinD1 (1:1,000) protein antibodies were added and incubated overnight at 4˚C. The films were then incubated with the secondary antibody (1:10,000) at room temperature for 1 h and washed three times with Tris-buffered saline/Tween 20 buffer. An enhanced chemiluminescence reagent (WBKLS0500; Merck Millipore, Billerica, MA, USA) was used to detect the protein levels, which were scanned using a Bio-Rad exposure system, and Image Lab 3.0 software used for quantification (Bio-Rad Laboratories, Inc.).

Immunofluorescence analysis. Daoy cells ($5x10^3$) were seeded on glass coverslips and treated with different concentrations of GANT61. At 24 h after incubation, the cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 1% Triton X-100 in PBS for 10 min. Next, the cells were incubated with rabbit anti-Gli1 and mouse anti-CyclinD1 antibodies at 37˚C for 1 h and washed with PBS. Subsequently, incubation for 1 h with DyLight594-conjugated goat anti-rabbit and FITC conjugated goat anti-mouse secondary antibodies (111-165-003 and 111-025-003; 1:10,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was performed, followed by DAPI staining. The cells were then mounted and observed under a fluorescence microscope.

Statistical analysis. SPSS version 19.0 (IBM Corp., Armonk, NY, USA) software was used for statistical analysis. Data were statistically analyzed by one-way analysis of variance. All experimental data are expressed as the mean ± standard deviation. $P<0.05$ indicated a statistically significant difference.

Results

Morphological changes of Daoy cells following GANT61 treatment. Daoy cells were cultured for 24 h, and then different concentrations of GANT61 (10, 20 or 40 μM in 0.1% DMSO) were added to examine the effects of GANT61 on the cell morphology. The cells were cultured for a further 24 h and then subjected to inverted microscopic observation. As shown in Fig. 1, the normal, non-adherent Daoy cells in the untreated control group were spherical in shape. Similarly, normal adherent cells were intercellular tight, follow flaky

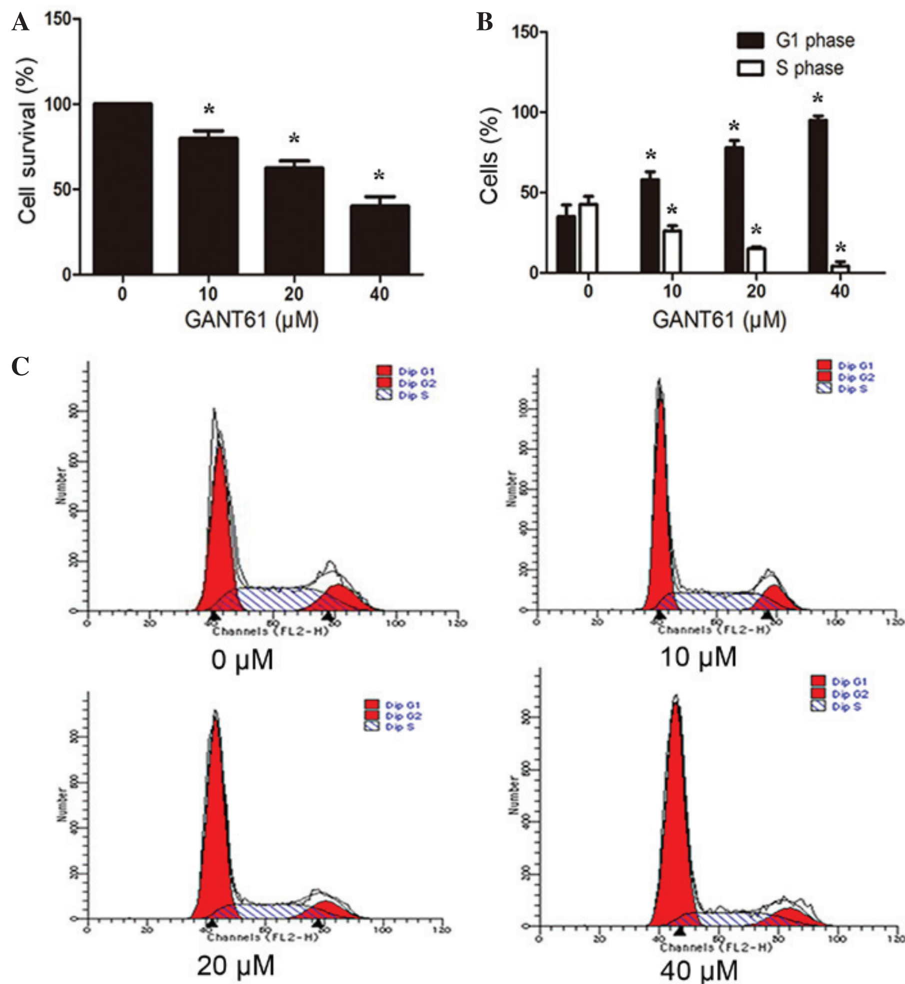

Figure 2. GANT61 inhibits the proliferation of Daoy cells and induces cell cycle arrest. (A) CCK-8 assay was used to investigate the effects of GANT61 treatment for 24 h on the survival of cells. GANT61 treatment inhibited the cell proliferation in a dose-dependent manner compared with the control group. (B) Percentage of cells at each cell cycle phase and (C) histograms of flow cytometry analysis, which was used to determine the effects of GANT61 treatment (0-40 μM in 0.1% dimethyl sulfoxide) for 24 h on cell cycle progression. GANT61 induced G1/S phase arrest of Daoy cells. Results are presented as the mean ± standard deviation of three independent experiments, and each sample was examined in triplicate (n=3). *P<0.05 vs. 0 μM group. CCK-8, cell counting kit-8.

aggregational growth and morphological rules, and their shapes were rectangular or triangular. Notably, groups treated with increasing concentrations of GANT61 demonstrated an evident decreased in cell number, as well as changes in morphology and diversity, which the cells presented with shrinkage and abnormal form. (Fig. 1).

GANT61 inhibits the proliferation and induces cell cycle arrest of Daoy cells. Marked morphological changes and decreased cell number was observed following GANT61 treatment (Fig. 1), indicating reduced cell proliferation or induced cell apoptosis. To elucidate whether cell proliferation was decreased following treatment with different concentrations of GANT61 for 24 h, the cell proliferation was detected by a CCK-8 assay. As shown in Fig. 2A, GANT61 significantly inhibited the proliferation of Daoy cells. The inhibition of proliferation in GANT61-treated groups compared with the control group was dose-dependent (P<0.05; Fig. 2A). Furthermore, to examine whether the growth inhibition of the cells was a result of cell cycle arrest, Daoy cells were stained with FITC-Annexin V and PI, and then subjected to flow cytometry. As displayed in Fig. 2B and C, the percentage of cells in G1 phase increased (P<0.05) with increasing concentration of GANT61 treatment, whereas cells in S phase decreased in a dose-dependent manner (P<0.05). This indicated that GANT61 resulted in cell cycle arrest of Daoy cells at the G1/S transition.

GANT61 promotes cell apoptosis of Daoy cells. To determine whether GANT61 treatment induced cells apoptosis, normal growing Daoy cells were treated with various concentrations of GANT61. After 24 h, the cells were subjected to HE staining and flow cytometry analysis. As shown in Fig. 3A, the HE staining results demonstrated that normal cells had a regular morphology. However, clearly visible abnormal morphologies were observed in Daoy cells treated with GANT61, with abnormal protuberance observed. The abnormal protuberance, chromatin condensation and fragmentation features were more evident at increased concentrations of GANT61, thus indicating a dose-dependent effect. HE staining also demonstrated decreased in cell number, increased cell shrinkage and nuclear fragmentation. As shown in Fig. 3B, the percentage of apoptotic cells increased significantly in the GANT61-treated cells, compared with the untreated group (P<0.05). These results

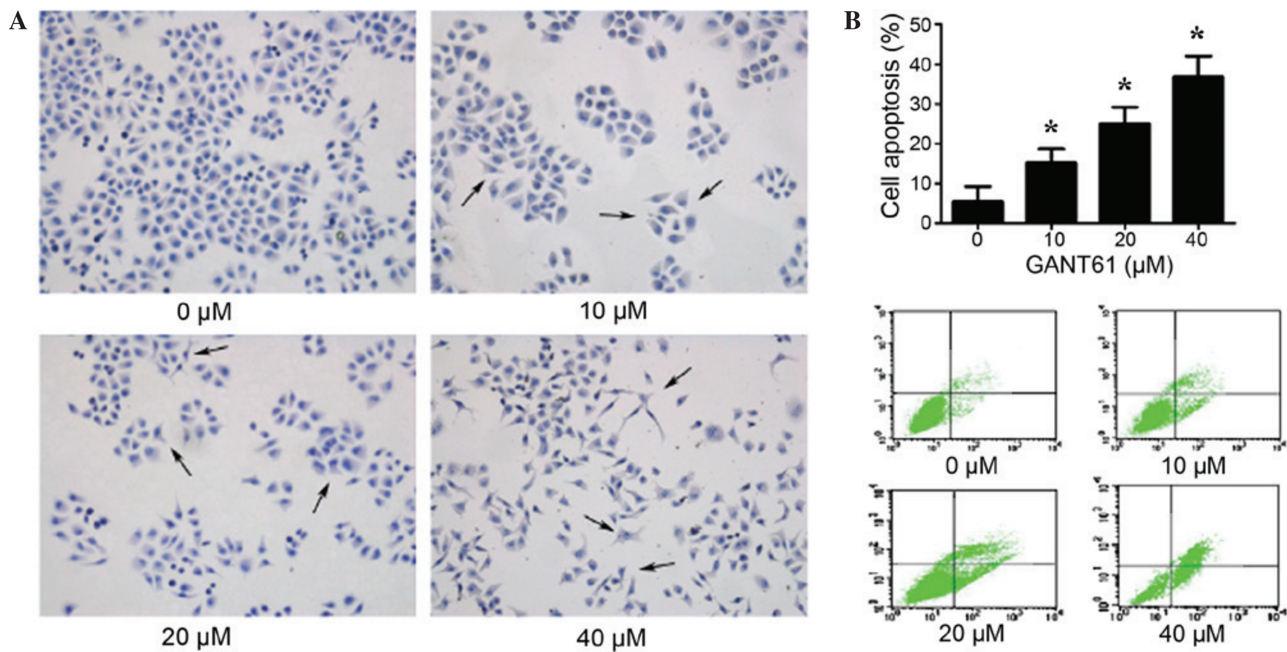

Figure 3. Cell apoptosis induces by GANT61 treatment for 24 h in Daoy cells. (A) Hematoxylin and eosin staining indicated increased coated abnormal protuberance with increasing concentrations of GANT61 (shown by arrows). (B) FITC-Annexin V flow cytometry analysis showed that GANT61 induced the apoptosis of Daoy cells in a dose-dependent manner. Experiments were performed at least three times (n=3). *P<0.05 vs. 0 μM group.

verified the prediction that GANT61 induced cell apoptosis in Daoy cells (19).

GANT61 inhibits the expression of Gli1 and CyclinD1 in the mRNA and protein level. To examine the underlying mechanism of reduced cell apoptosis and cell cycle arrest, the total RNA of the cells were extracted by TRIzol reagent, reverse transcribed into cDNA and then subjected to PCR. Gli1 is an important transcription factor in the SHH signaling pathway, regulating the transcription of multiple downstream target genes, including CyclinD1, the oncogene controlling cell cycle entry (22,23). As shown in Fig. 4A, the results revealed that GANT61 was able to significantly inhibit the gene expression of Gli1 (P<0.05). Along with the decreased expression of the Gli1 gene, CyclinD1 mRNA appeared to be downregulated synchronously (P<0.05). In addition, protein levels were assayed by immunofluorescence analysis. As indicated in Fig. 4B and C, CyclinD1 was mainly localized in the cytosol of Daoy cells, whereas Gli1, as a transcription factor, was located in both the cell cytosol and nucleus. Following treatment with GANT61 for 24 h, Daoy cells showed decreased levels of Gli1 protein compared with that in untreated cells (P<0.05). Subsequently, CyclinD1 was also decreased, as one of the Gli1 transcriptional targets (P<0.05). The inhibition by GANT61 on Gli1 and CyclinD1 was dose-dependent. To further elucidate the inhibitory effects of GANT61 on the expression of Gli1 and CyclinD1, their protein levels were examined by western blot analysis. Daoy cells treated with GANT61 for 24 h were lysed and separated by SDS-PAGE, and the protein expression levels of Gli1 and CyclinD1 were detected using the corresponding antibodies. The results demonstrated that GANT61 was able to decrease the level of Gli1 protein (Fig. 5). In line with the decreased expression of Gli1 protein, CyclinD1 protein also appeared to be downregulated (P<0.05). The inhibition of Gli1 and CyclinD1 protein levels by GANT61 was in a dose-dependent manner (P<0.05). These results were consistent with the data obtained by qPCR and immunofluorescence analyses, indicating that GANT61 can significantly inhibit Gli1 and CyclinD1 expression at the mRNA and protein levels.

Discussion

Aberrant activation of the SHH signaling pathway is implicated in various types of human cancer (24). The SHH signaling pathway is important in regulating cell proliferation and differentiation in the embryonic development of the cerebellum (25). MB is characterized by constitutive activation of the SHH signaling pathway, and is genetically characterized by mutations in patched homolog 1 (PTCH1), which blocks the function of smoothened (SMO), or other downstream pathway mutations (26). Gli1 expression is inhibited by suppressor-of-fused, preventing it from activating gene transcription. The binding of SHH to PTCH1 or other mutations releases a basal repression on SMO, which is then activated (27). Subsequently, Gli1 is released and activates a series of gene transcriptions (28,29).

Inhibitors of the SHH signaling pathway are currently being developed to mainly target SMO or its upstream sites (30). Numerous studies using such inhibitors in MB have demonstrated the efficacy of this treatment, and these findings have been translated into Phase I and II clinical trials (31-34). While these therapies have shown promising results, various significant challenges remain, including the possible long-term bone marrow suppression and drug toxicity (35,36). As the majority of targeted therapies for MB have focused on SMO, it is concerning that only a single mechanism has been identified and targeted, making resistance a frequently encountered complication (37). SMO mutation is not the only mechanism

Figure 4. GANT61 inhibits the expression of Gli1 and CyclinD1 in Daoy cells treated with different GANT61 concentrations for 24 h. (A) mRNA expression levels of Gli1 and CyclinD1, as determined by quantitative polymerase chain reaction. Gli1 and CyclinD1 mRNA levels were downregulated following GANT61 treatment, indicating that GANT61 selectively inhibited the SHH signaling pathway at the mRNA level. (B) Mean density and (C) images of immunofluorescence analysis, investigating the protein levels of Gli1 and CyclinD1 to determine the effect of GANT61 treatment on the SHH signaling pathway at protein level. CyclinD1 was mainly localized in the cytosol of Daoy cells, while Gli1 extended to the cell cytosol and nucleus. Gli1 and CyclinD1 were downregulated, and the inhibition was reduced by GANT61 treatment in a dose-dependent manner. *P<0.05 vs. 0 μM group. Analyses were performed at least in triplicate for each experiment (n=3). SHH, sonic hedgehog; Gli1, Gli family zinc finger 1.

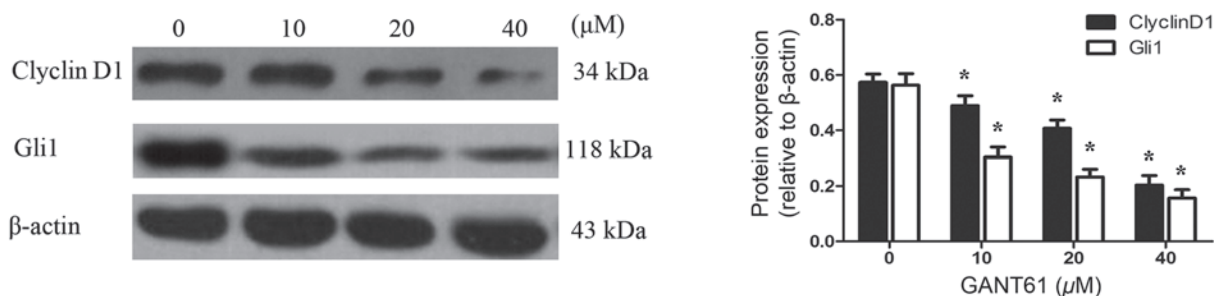

Figure 5. GANT61 selectively inhibits SHH signaling pathway at the protein level, as determined by western blot analysis. Gli1 and CyclinD1 were downregulated by GANT61 treatment. Analyses were performed at least in triplicate for each experiment (n=3). *P<0.05 vs. 0 μM group. SHH, sonic hedgehog; Gli1, Gli family zinc finger 1.

of acquired drug resistance, as the development of other downstream hedgehog pathway component mutations have since been implicated in SHH inhibitor resistance. Kool *et al* (38) indicated that amplifications of Gli may result in inability to respond to current SMO inhibitors. Such aberrations include the amplification of Gli and the upregulation of PI3K/AKT signaling, manifesting *in vivo* as tumor regrowth in the same mouse model (39,40).

Gli1 serves a crucial role in the transformation and proliferation of malignant cells (41). It is also important for preventing apoptosis and maintaining the malignant proliferation of tumor cells, and is involved in tumor cell protection against chemotherapy (42). Berman *et al* (43) indicated that the expression level of Gli1 may reflect the degree of activation of the SHH signaling pathway. Inhibition of abnormal activation of this signaling pathway by inhibiting the expression of Gli1 can inhibit the growth of tumor cells. Gli1, as the main transcription factor downstream of the SHH signaling pathway, may be able to inhibit tumor cell proliferation and differentiation through downregulation of downstream target genes. On the basis of the pivotal role of Gli1 in malignant cells, it has become increasingly evident that Gli1 is a promising target for anticancer therapy. A direct strategy to interfere with Gli1 activity is to induce selective inhibition of its DNA transcription.

GANT61, an agent that exerts an inhibitory activity of the SHH signaling pathway, functions by selectively binding to Gli1 and has been found to suppress proliferation in various tumors (44,45). In the present study, GANT61 had *in vitro* activity against tumor proliferation, and induced cell cycle arrest and apoptosis. Furthermore, GANT61 was found to inhibit the Gli1 mRNA and protein expression levels. Dysregulation of cell cycle progression is considered to serve an important role in cancer; thus, the current study investigated whether Gli1 is associated with the typical oncogene CyclinD1 in the cell cycle. CyclinD1 is a key protein regulating the G1/S transition in the cell cycle and is highly expressed in multiple types of tumors (46). CyclinD1 is frequently deregulated in various cancer types, and is a biomarker of cancer phenotype and disease progression (46,47). Overexpressed CyclinD1 accelerates the cell cycle transition, leading to uncontrolled cell proliferation and the development of cancer. The present study identified that the mRNA expression of Gli1 was significantly associated with CyclinD1 expression in MB, and a similar observation was identified regarding the protein levels. Suppressing the expression of Gli1 may inhibit the overexpression of CyclinD1 and the proliferation of tumor cells, and synchronously promote cell apoptosis. Therefore, blocking the expression of Gli1 may be an attractive therapeutic strategy for MB.

In conclusion, SHH signaling pathway can regulate tumor cell cycle and apoptosis in different molecular levels. Increased expression of Gli1 induced the upregulation of CyclinD1 expression, thus promoting cell proliferation, which may be one of the growth patterns of tumor cells. Therefore, Gli1 may be an important target for MB treatment. Therapies using Gli1-targeted inhibitors alone or combined with other cytotoxic chemotherapeutics may become an effective targeted treatment of MB. However, the association of the SHH signaling pathway and other pathways in MB cells with the specific mechanism of apoptosis induced by targeted therapy requires further investigation.

Acknowledgements

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